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The Effect of *Pedilanthus tithymaloides* (L.) Poit Crude Extract on Wound Healing Stimulation in Mice

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ABSTRACT

The study aimed to investigate the potential of an ethanolic extract of *Pedilanthus tithymaloides* to stimulate excision wound healing. Samples of 42 female mice were divided into three groups: group I – methylcellulose (negative control); group II – 0.5% (w/w) crude extract; and group III – 1.5% (w/w) crude extract. The results were compared among treatments and a control group, in terms of wound appearance, parameters of wound healing and a histopathological study. In the treatment groups, the parameters of wound healing were significantly ($P < 0.05$) higher in comparison with the control group. The histopathological study also showed increased fibroblast, collagen fiber and blood vessel formation. However, the wounds treated by 0.5% crude extract healed faster when compared to the group with 1.5% crude extract. In conclusion, 0.5% crude extract of *P.tithymaloides* was more suitable for the stimulation of wound healing than 1.5% crude extract because the latter was too concentrated that caused irritation and inflammation, leading to delayed healing.

Keywords: *Pedilanthus tithymaloides*, wound healing, excision wounds, ethanolic crude extract, mouse

INTRODUCTION

External wound healing is a complicated process with four important stages that occur continuously. Hemostasis occurs immediately at the time of injury to stop bleeding by platelet aggregation and platelet-mediated vasoconstriction. In the second stage (inflammation), the injured tissue cells and capillaries activate the release and function of several cytokines which induce phagocytosis to remove debris and initiate wound repair. In the third proliferative stage, the wound surface is covered with a new epithelium (epithelization) and granulation tissues with new vascularization are formed to repair the injured tissue. The final stage, remodeling, is responsible for balancing the new collagen synthesis and the degradation of old tissues (Strodtbeck, 2001). Although the process progresses naturally, the healing of deep and wide wounds can be seriously delayed. Thus medicines which contain antibiotics and anti-inflammatory agents have been used for rapid healing, by reducing infection and inflammation. Several plant extracts, for example, *Aloe vera* and *Centella asiatica*, are also included in the formulation of some medicines due to the well-known anti-inflammatory activity of these plants (Singhabuttra, 1993). However, many Thai native plants have been used to cure wounds, but have not been studied for their activity on wound healing stimulation.

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P. tithymaloides is a medicinal plant applied to wounds for rapid healing (Sripirom, 1981). The chemical analysis of stems and leaves from *P. tithymaloides* has identified kaempferol 3-*O*-β-D-glucopyranoside-6-(3-hydroxy-3-methylglutarate), quercitrin, isoquercitrin and scopoletin, which are phenolic and flavonoid compounds (Abreu *et al*., 2008). Previous studies reported an anti-inflammatory effect of the stem and leaves of *Pedilanthus tithymaloides* in rats that induced paw edema by carageenan. The intraperitoneal injection of ethanolic extract at levels of 500, 750 and 1,000 mg/kg body weight reduced inflammation by about 83, 94 and 92%, respectively. In addition, an in vitro study using the scavenging activity method, expressed as an inhibitory concentration (IC_{50}), showed that the extract could efficiently inhibit ROS (reactive oxygen species) and RNS (reactive nitrogen species) (Abreu *et al*., 2006). The chemical constituents isolated from *P. tithymaloides* studied by the broth microdilution method using Mueller-Hinton and RPMI-1640 media showed strong inhibition against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli* (Vidotti *et al*., 2006). This work indicated that the antioxidant property of *P. tithymaloides* stimulated anti-inflammation and anti-bacterial activity, which supported rapid healing of the wound.

In the present study, the effect of *P. tithymaloides* crude extract on wound healing stimulation was evaluated by wound appearance, wound closure time and the histopathology of the wound. Although this plant has been used as a medicinal plant to heal wounds, the supporting scientific data has not been reported. This work was expected to benefit the development of a herbal medicine to treat serious wounds that require rapid healing, and to increase value of Thai native plants.

MATERIALS AND METHODS

Preparation of plant extract

Fresh leaves of *P. tithymaloides* were collected from Sing Buri province in July 2008. The plant was cut into small pieces and extracted with 95% ethanol for 48 h. Then, ethanol was evaporated using a rotary evaporator. The semi-solid crude extract was mixed with methylcellulose to final concentrations of 0.5 and 1.5%. Methylcellulose alone was used as a control.

Animals

Forty two female ICR mice (established at the **I**nstitute fore **C**ancer **R**esearch in 1947) aged 8 w (weight, 25-30 g) with no prior drug treatment were used for all studies. The animals were fed with a commercial pellet diet and water ad libitum and acclimatized to laboratory conditions for 10 d before starting the experiments. All mice were divided into three groups, each of which consisted of 14 animals: group I – methylcellulose (negative control); group II – 0.5% crude extract; and group III – 1.5% crude extract.

Excision wound model

The animals in each group were anaesthetized by pentobarbital sodium (50-60 mg/kg, IP) (Kullaprawit, 2001), shaved and sterilized on the dorsal area. An excision wound was made by cutting away 1cm x 1cm full thickness skin on the dorsum between the shoulders. The methylcellulose, 0.5% extract and 1.5% extract were applied daily on the wound surface of mice in group I, II and III, respectively, until the wound was completely healed. During the healing period, the total wound area and the open wound area were measured on six mice in each group at 2-days intervals. The wound specimens were collected from two mice on day 4, 8, 12 and 16 for the histopathological study.

Parameters of wound healing

The total wound area and open wound area were measured by tracing the wound margin onto a transparent sheet and then placing the sheet onto scaled graph paper. The subtraction of the open wound area from the total wound area was used to estimate the area of epithelium. The parameters of wound healing were calculated by formulae from Bohling *et al.,* 2006 (Figure 1).

Histopathological study

Sample tissues were fixed in Bouin's fluid and embedded in paraffin. Serial sections (5 μm thickness) of paraffin-embedded tissue were cut. Tissue samples were stained with H&E and Masson's trichrome and examined by light microscope to observe morphology, the number of fibroblasts, collagen deposition, angiogenesis and epithelization.

Statistical analysis

Data were collected on the percentages of epithelization, wound contraction and total wound healing in the mice that were treated with 0.5 and 1.5% *P. tithymaloides*. Analysis was undertaken using PROC GLM from the statistical analysis software developed by the SAS Institute Inc. P values of $P < 0.05$ were considered statistically significant.

RESULTS

Wound appearance

On the first day, all wounds were dry and had a large amount of scab. Thereafter, wounds became moist and granulation tissue appeared. Swelling and bleeding occurred in the wounds treated with the 1.5% crude extract until day 4. Meanwhile, the open wound sizes of the other wounds were markedly reduced. The wounds treated with 0.5 and 1.5% crude extract were completely healed at the end of the second week. In the control group, the methylcellulose treated wounds were the slowest to heal, based on the parameters of wound healing (Tables 1-3) mentioned below.

$$\textbf{\% epithelization at day}_n = \frac{\text{area of epithelium at day}_n}{\text{total wound area at day}_n} \times 100$$

% wound contraction as:

$$\text{step 1: total wound at day}_n \text{ as \% of original} = \frac{\text{total wound area at day}_n}{\text{original wound area (day}_0\text{)}} \times 100$$

$$\text{step 2: \%wound contraction at day}_n = 100 - \text{total wound at day}_n \text{ as \% of original}$$

% total wound healing as:

$$\text{step 1: open wound at day}_n \text{ as \% of original} = \frac{\text{open wound area at day}_n}{\text{original wound area (day}_0\text{)}} \times 100$$

$$\text{step 2: \%total wound healing at day}_n = 100 - \text{open wound at day}_n \text{ as \% of original}$$

Figure 1 Formulae used to calculate the parameters of wound healing.

Parameters of wound healing

The wounds treated with 0.5% crude extract (group II) showed higher percentages for average epithelization, wound contraction and total wound healing throughout the study (Tables 1-3). The percentage of epithelization in group II at day 10 was significantly higher than the other groups (47.09, 74.01 and 49.01% in groups I, II and III, respectively) and it was continuously higher until day 12 (56.37, 93.89 and 70.37% in groups I, II and III, respectively). At day 14, the epithelization of wounds treated by 0.5 and 1.5% crude extracts (groups II and III) reached 100%, whereas the methylcellulose treated wounds showed only 80.84% (Table 1).

The wound contraction of group II trended to be higher than other groups at all time intervals. However, it was significantly higher only at day 4 (22.16, 36.36 and 5.93% in groups I, II and III, respectively) and day 6 (34.84, 47.08 and 30.74% in groups I, II and III, respectively) (Table 2).

The total wound healing of group II also showed a better trend than other groups. However, the significantly higher values of the total wound healing in group II was revealed at days 4, 6, 10 and 12. At day 14, the total wound healing of groups II and III reached 100% whereas group I showed only 96.50% (Table 3).

Table 1 Percentage** of wound epithelization in groups treated with crude extract and the control group.

Treatment	Time (d)								
	0	2	4	6	8	10	12	14	16
I Methylcellulose	0	0	0	0^{b}	23.31±4.37^{b}	47.09±20.57^{b}	56.37±23.44^{b}	80.84±16.04^{b}	100
II 0.5% crude extract	0	0	0	11.11±5.59^{a}	53.51±16.76^{a}	74.01±10.19^{a}	93.89±10.15^{a}	100^{a}	100
III 1.5% crude extract	0	0	0	10.86±3.35^{a}	34.49±17.72^{ab}	49.01±16.05^{b}	70.37±5.83^{b}	100^{a}	100

Each value represents mean ± standard deviation, $n = 6$.

**Means with different superscripts within the same column were significantly different at *P-value* < 0.05, using Proc GLM in the SAS statistical package.

Table 2 Percentage** of wound contraction in crude extract treated groups and the control group.

Treatment	Time (d)								
	0	2	4	6	8	10	12	14	16
I Methylcellulose	0	13.60±6.01^{a}	22.16±5.01^{b}	34.84±5.42^{b}	49.00±6.19^{a}	64.72±8.68^{a}	73.97±7.02^{b}	82.31±5.31	82.67±4.93
II 0.5% crude extract	0	20.86±3.22^{a}	36.36±5.18^{a}	47.08±5.58^{a}	58.76±7.24^{a}	72.46±5.84^{a}	82.28±3.71^{a}	84.17±2.85	84.29±2.97
III 1.5% crude extract	0	-6.65±13.58^{b}	5.93±13.77^{c}	30.74±16.33^{b}	50.49±9.08^{a}	67.80±6.65^{a}	76.26±2.71^{ab}	80.50±3.44	80.50±3.44

Each value represents mean ± standard deviation, $n = 6$.

**Means with different superscripts within the same column were significantly different at *P-value* < 0.05, using Proc GLM in the SAS statistical package.

Table 3 Percentage** of total wound healing in crude extract treated groups and control group.

Treatment	Time (d)								
	0	2	4	6	8	10	12	14	16
I Methylcellulose	0	13.60±6.01^{a}	22.16±5.01^{b}	34.84±5.42^{b}	60.77±6.21^{b}	82.42±4.67^{b}	89.18±4.91^{b}	96.50±2.88^{b}	100
II 0.5% crude extract	0	20.86±3.22^{a}	36.36±5.18^{a}	52.88±6.60^{a}	79.89±10.33^{a}	92.38±4.57^{a}	98.66±2.21^{a}	100^{a}	100
III 1.5% crude extract	0	-6.65±13.58^{b}	5.93±13.77^{c}	38.35±9.63^{b}	67.90±9.11^{ab}	83.79±5.69^{b}	92.96±1.58^{b}	100^{a}	100

Each value represents mean ± standard deviation, $n = 6$.

**Means with different superscripts within the same column were significantly different at *P-value* < 0.05, using Proc GLM in the SAS statistical package.

Histopathological study

The H&E stained sections at day 4 showed that the wound surfaces of all groups were covered by tissue debris containing blood and leukocytes indicating the inflammatory process, especially in wounds of groups I and III. The formation of granulation tissue was first observed on day 8, with the display of pinkish homogenous substances with fine collagen fibrils in all wounds, but was complete in group II. On day 12, all wounds were closed and granulation tissue began to contain collagen fibers. The fibroblasts and small blood vessels were found throughout the granulation tissues of wounds treated with the 0.5 and 1.5% crude extracts, but were found scarcely in the control. Stellate-shaped fibroblasts existed in wounds treated with the 1.5% crude extract, but there were large numbers of cells in the wounds treated with the 0.5% crude extract (Figure 2). Based on histological observations, most wounds in all groups were completely healed by day 16. However, staining with Masson's trichrome showed that the collagen bundles formed in the granulation tissue of group II were larger than those found in group I and III samples. Randomized measurement from 50 collagen bundles showed average bundle sizes of 1.38, 5.86 and 3.64 μm in groups I, II and III, respectively (Figure 3).

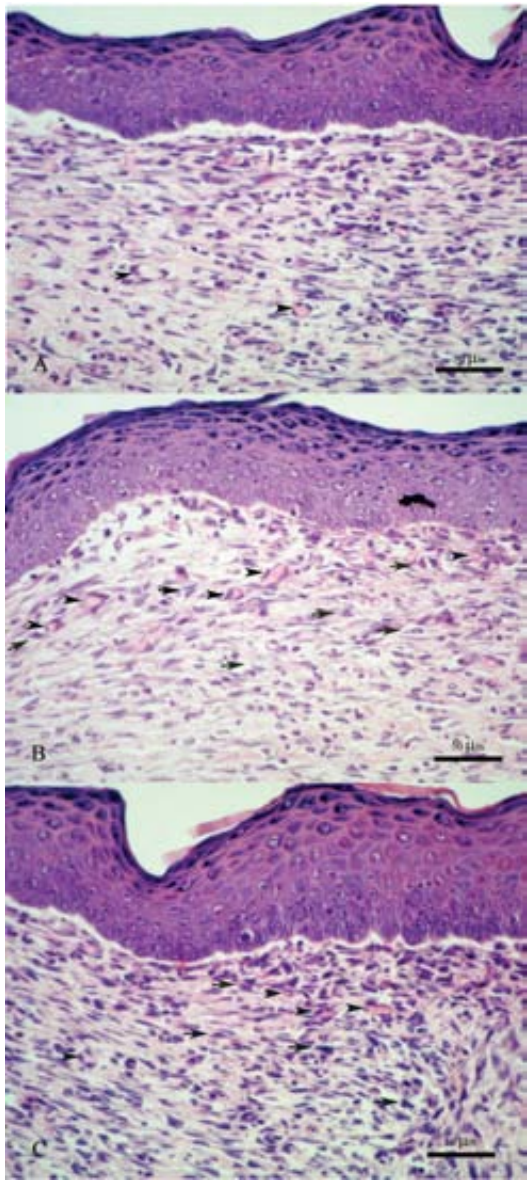

Figure 2 H&E-stained section of granulation tissue at day 12 (40X):
(A) group I (control); (B) group II (0.5% crude extract); and (C) group III (1.5% crude extract). Arrow head (>) = small blood vessel; Arrow (→) = stellate-shaped fibroblast.

DISCUSSION

In the first week of healing, all wounds still showed signs of swelling and bleeding, especially the wounds treated by methylcellulose and the 1.5% crude extract. This result corresponded to the histopathological study which showed many small blood vessels and leukocytes. This feature indicated that the wounds were in the inflammatory stage that led to delayed wound closure (Strodtbeck, 2001). The study of wound healing in the second week displayed granulation tissues which contained pinkish homogenous substances, including thin fibrils. The constituents of this structure are made up of glycosaminoglycans (GAGs) (mainly hyaluronan), glycoprotein,

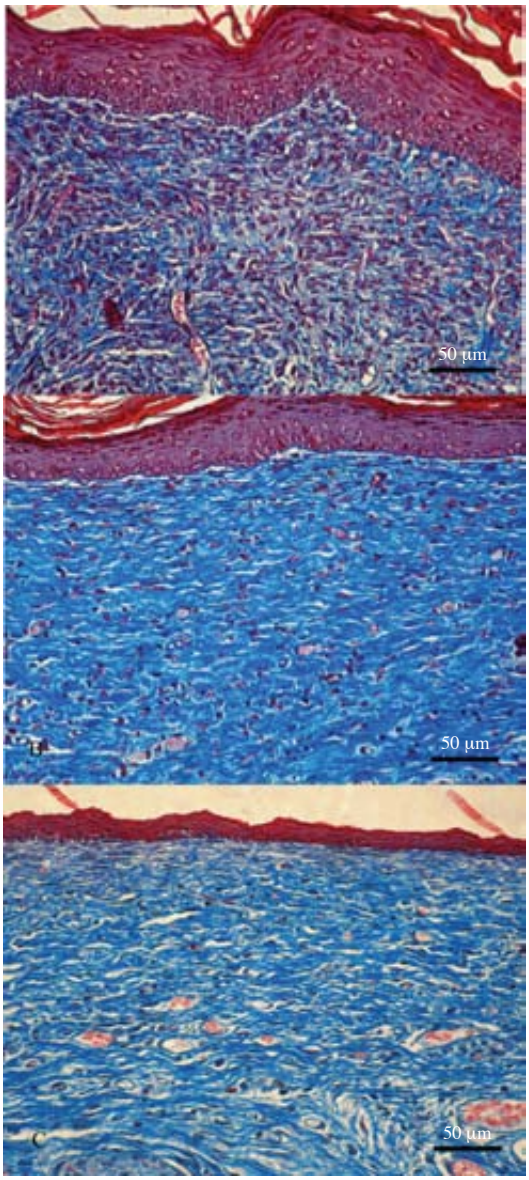

Figure 3 Masson's trichrome stained sections of the granulation tissue at day 16 (40X): (A) group I (control); (B) group II (0.5% crude extract); (C) group III (1.5% crude extract).

proteoglycan, fibronectin and collagen types I, III and V (Hering *et al.*, 1983; Chen, 1999; Strodtbeck, 2001). This process exhibited the initiation of the proliferative stage. In addition, fibroblasts and small blood vessels were found throughout the granulation tissue of the wounds treated with the 0.5 and 1.5% crude extracts, but the wounds treated with the 0.5% crude extract had many stellate-shaped fibroblasts which were believed to be myofibroblasts. However, additional techniques, such as immunohistochemistry, would be required to confirm the α-smooth muscle actin expressed in these cells (Hinz, 2005). Such a structure helps the myofibroblast to contract the wound and reduce the wound area (Carlson and Longaker, 2004; Li and Wang, 2009). The histopathological finding in the present study indicated that the 0.5% crude extract stimulated wound closure and enhanced wound healing which was correlated to the parameters of wound healing (Tables 1-3). Moreover, the maturation of collagen bundles in this group was more developed than in others, as observed by Masson's trichrome staining.

CONCLUSION

The 0.5% crude extract of *P. tithymaloides* was more suitable for stimulating wound healing than the 1.5% crude extract, because it produced increased percentages of average epithelization, wound contraction and total wound healing and the wound appearance was fresh and moist. The latex of *P. tithymaloides* contains toxic phorbol derivatives, namely euphorbol, caoutchouc, resin and pedilstatin (Buckingham, 1994; Pettit *et al.*, 2002). The high concentration of these toxins caused wound irritation and inflammation which induced slower epithelization and delayed wound healing (Strodtbeck, 2001).

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